Localization of ZnT7 and zinc ions in mouse retina— Immunohistochemistry and selenium autometallography

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Abstract

Zinc transporter 7 (ZnT7, Slc30a7), a member of the Slc30 family, is involved in mobilizing zinc ions from the cytoplasm into the Golgi apparatus. In the present study, we examined the distribution and localization of ZnT7 and the labile zinc ions in the mouse retina using immunohistochemistry and in vivo zinc–selenium autometallography (ZnSe^{AMG}). Our results showed that ZnT7 is abundantly expressed in the ganglion cells and pigment epithelial cells of the mouse retina. ZnT7 is also expressed in the amacrine cells and the layer of optic fibers of the mouse retina, but to a lesser extent. Weak staining of ZnT7 was detected in the inner plexiform layer, outer plexiform layer, and outer segment of the photoreceptors. However, ZnT7 was not detected in the outer nuclear layer and inner segment of the photoreceptors. A high level of labile zinc pool was detected in the pigment epithelial cells, the inner segment of the photoreceptors, and the marginal region of the inner nuclear layer. Less amount of labile zinc ions were detected in the ganglion cells of the retina. These observations strongly suggest that ZnT7 may play critical roles in retinal zinc homeostasis and that chelatable zinc pools may have multiple functions in the retina.
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1. Introduction

Zinc is an essential trace metal which plays critical roles in biological processes of the body. In brain, approximately 90% of total zinc is tightly bound in metalloenzymes and other zinc-containing proteins, where zinc serves as a cofactor for enzymatic activities or for maintaining the three-dimensional structure of proteins [6,4]. The remaining 10% of total zinc is called chelatable zinc, because it presents as free or loosely bound ions. These chelatable zinc ions, most of which are localized in a population of synaptic vesicles of the so-called zinc enriched (ZEN) terminals, can be detected by both fluorescence and autometallographic (AMG) techniques. The fluorescence techniques including TSQ and Zinquin staining are suitable for low magnification approaches while the AMG techniques are excellent for detecting nM levels of chelatable zinc ions in tissues [17,10,51,16,9].

The telencephalon contains large amount of zinc enriched neuronal somata and terminals. Neocortex and hippocampal regions of brain contain the highest zinc enriched neuronal terminals in the human brain. These zinc enriched neuronal terminals are glutaminergic [31,32,12]. During synaptic vesicle exocytosis, vesicular zinc is co-released with glutamate into the synaptic space [3,21,2]. It has been suggested that these zinc ions modulate the activities of the excitatory *N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors localized on the postsynaptic membrane ([49,35]). In addition, studies also showed that most zinc enriched terminals in the spinal cord and cerebellum are either GABAergic or glycinergic [44–46].

Zinc is abundant in the mammalian retina [20]. Several studies have demonstrated that cellular zinc ions were localized in the inner segment of the photoreceptors, the outer and inner plexiform layers, and inner nuclear layer [1]. However, the ultrastructural evidence of the exact zinc localization is

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not strong. These shortcomings make the final proof of where the zinc ion pools are localized incomplete which obscures understanding of the biological roles of zinc in the retina [14,15,18–20,23–26,33,34,40–42,50]).

Recent studies have indicated that the zinc transporter (ZnT) family plays critical roles in maintaining intracellular zinc homeostasis [7,29]. Since 1995, eight members in ZnT family, namely ZnT1-8, have been cloned and functionally characterized. All members analyzed so far have similar membrane topology with six transmembrane domains and a histidine-rich loop between domains IV and V, where zinc has been presumed to be bound by histidines and subsequently transported across the membrane. Although ZnT1-8 proteins resides in different subcellular compartments, they function as zinc efflux transporters to reduce the cytoplasmic zinc concentrations by transporting zinc directly out of the cell or into intracellular compartments [27]. The detailed distribution of ZnT3 in the light-adapted mouse retina has been described using immunohistochemical techniques. Immunostaining indicated that the ZnT3 protein is abundant in the regions of the outer limiting membrane and the inner segment of photoreceptors. The ZnT3 immunoreactivity was also found in the outer plexiform, inner nuclear, inner plexiform, and ganglion cell layers [36]. It is well known that ZnT3 is localized on the synaptic vesicle membrane and is required for transporting zinc into synaptic vesicles from cytosol [28,48,5,11]). The abundant expression of ZnT3 in the retina suggests that ZnT3 may be important for synaptic zinc transportation and for maintaining zinc metabolism in the mouse retina.

ZnT7 is abundantly expressed in the mouse small intestine and lung [27]. Double immunofluorescent labeling has demonstrated that the ZnT7 protein resides in the Golgi apparatus as well as an unknown vesicular compartment. Over expression of ZnT7 in Chinese hamster ovary (CHO) cells induced accumulation of zinc in the Golgi apparatus when cells were exposed to a higher concentration of zinc [27,13,22,29]. In this study, we examined the distribution and localization of ZnT7 and chelatable zinc ions in the mouse retina to elucidate the retinal zinc physiology.

2. Materials and methods

2.1. Experimental animals

Adult male CD-1 mice, weighing approximately 40–50 g, were used as experimental animals in this study. They were housed in a 12 h light/dark cycle with food and tap water available ad libitum. All experimental procedures were performed in an agreement with the ethical standards of China Medical University.

2.2. Immunohistochemical staining for ZnT7

Three adult male CD-1 mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 50 ml isotonic saline, followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The entire eye balls were removed and postfixed using the same fixative at 4 °C for 4 h.

The fixed eye balls were placed in a 30% sucrose solution made in PB at 4 °C overnight. Anteroposterior axes sections (10 μm) of retinas were prepared on a cryostat and placed on glass slides. An affinity-purified rabbit anti-ZnT7 polyclonal antibody was used for immunohistochemical detection of the ZnT7 protein in the retina sections [27]. The immunostaining procedures were per-

formed in accordance with the standard ABC method. Briefly, sections were rinsed in 0.1 M Tris-buffered saline (TBS, pH 7.4) and endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide (H_2O_2) in pure methanol for 10 min. Sections were rinsed three times in TBS and treated with 5% bovine serum albumin (BSA) and 3% goat serum in TBS for 1 h to reduce nonspecific staining. Sections were then rinsed in TBS for 30 min and incubated in TBS containing 3% goat serum, 1% BSA, 0.3% Triton X-100, and 1:100 diluted anti-ZnT7 antibody at 4 °C overnight. After rinsing in TBS, sections were incubated in biotinylated goat anti-rabbit IgG (1:200) at room temperature for 1 h. The ABC Kit (DAKO) was applied at room temperature for 1 h in order to visualize the immunoreaction sites. Color was developed by rinsing sections in 0.1 M Tris buffer (pH 7.6) and incubating sections in 0.025% 3,3-diaminobenzidine (DAB) with 0.0033% H_2O_2 at room temperature for 10 min. Sections were then rinsed with Tris buffer to stop the DAB reaction. Some sections were counterstained with 1% toluidine blue. All sections were mounted and examined under a light microscope equipped with an image analysis system.

Control sections were incubated with normal sera instead of the primary anti-ZnT7 antibody followed by all subsequent incubations as described above. No distinct staining was observed.

2.3. Zinc–selenium autometallography

Three mice were used for analyzing the distribution of chelatable zinc ions by zinc–selenium autometallography in the mouse retina. Deeply anesthetized mice (50 mg/kg pentobarbital) were injected intraperitoneally with sodium selenite (30 mg/kg) and kept in cages for 40 min. Animals were then perfused transcardially with 100 ml isotonic saline, followed by 100 ml 2.5% glutaraldehyde in 0.1 M PB. The entire eye balls were removed and immediately immersed in the same fixative at 4 °C for 4 h.

For cryoprotection, all samples were placed into 30% sucrose at 4 °C overnight. After frozen, 10-μm-thick cryostat sections were cut and placed on glass slides pre-treated with Farmer solution in order to get rid of contamination. Sections were incubated in the AMG developer in a 26 °C water bath for 1 h as described by Danscher ([8]). The AMG developer consists of 60 ml gum Arabic, 10 ml citrate buffer, 0.85 g hydroquinone in 15 ml distilled water, and 0.12 g silver-lactate in 15 ml distilled water. After AMG developer incubation, sections were immersed in a 5% thiosulphate solution to stop the AMG reaction. Sections were then placed under running tap water (37–40 °C) for 20 min in order to remove the gelatine membrane, dipped in distilled water and counterstained with 1% toluidine blue. The sections were analyzed and photographed with a light microscope.

3. Results

3.1. Expression of ZnT7-immunoreactivity in the mouse retina

Seven layers above the pigment epithelial layer (PEL) were distinguished in the mouse retina sections incubated with an anti-ZnT7 antibody (Fig. 1a). They were: (1) the photoreceptor layer (OS and IS), (2) the outer nuclear layer (ONL), (3) the outer plexiform layer (OPL), (4) the inner nuclear layer (INL), (5) the inner plexiform layer (IPL), (6) the ganglion cell layer (GC), and (7) the nerve fiber layer (NFL).

The somata of the ganglion cell layer and the pigment epithelial layer showed the strongest ZnT7 immunoreactivity (Fig. 1; Table 1). The nerve fiber layer and inner nuclear layer exhibited moderate ZnT7 reactions (Fig. 1a; Table 1), suggesting that both the axons and dendrites of ganglion cells contain ZnT7. A moderate reaction of ZnT7 was also observed in the outer segment (OS) but not in the inner segment (IS) of the photoreceptors (Fig. 1a and d; Table 1). The inner plexiform layer and outer plexiform layer showed weak ZnT7 reactions (Fig. 1a; Table 1).
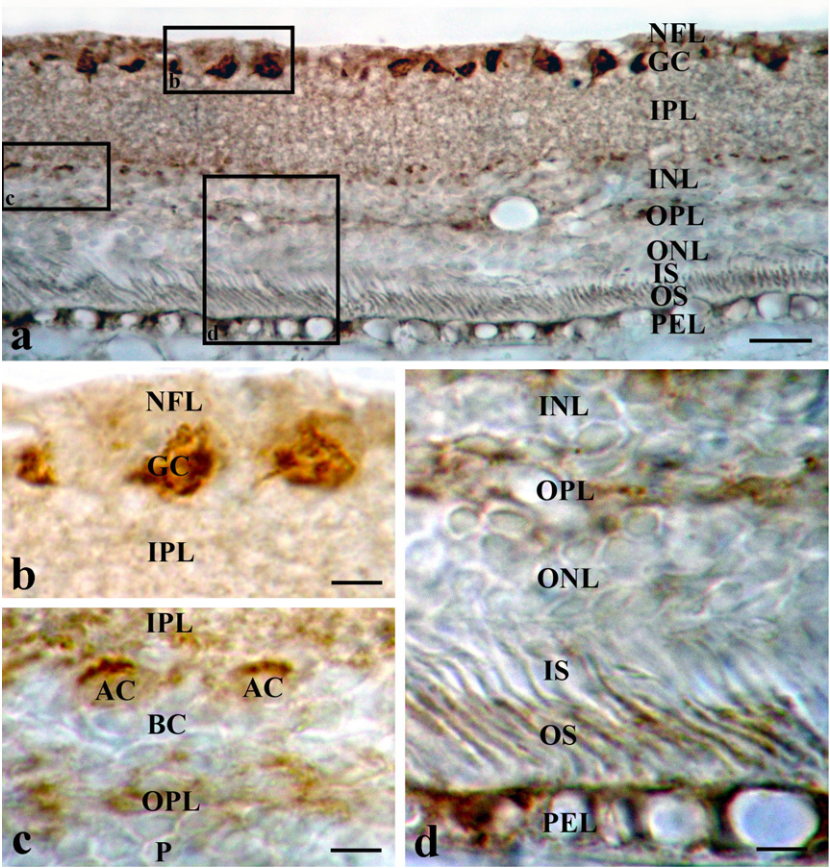
Fig. 1. Immunohistochemical localization of ZnT7 in the mouse retina. (a) Strong staining for ZnT7 was detected in the cell bodies of ganglion cells (GC) and pigment epithelial cells (PEL). Moderate immunostaining was observed in the optic nerve fiber layer (NFL), near the marginal regions of the inner nuclear layer (INL), and outer segment of photoreceptors (OS). Weak ZnT7 staining was seen in the inner plexiform layer (IPL) and outer plexiform layer (OPL). (b) High magnification showed that the strong ZnT7 immunostaining is predominately located in the perinuclear areas. (c) ZnT7 staining is detected in the amacrine cells (AC) in the inner nuclear layer. Faint ZnT7 immunostaining was seen in the inner plexiform layer (IPL) and the outer plexiform layer (OPL). No distinct immunostaining was observed in the cytoplasm of bipolar cells (BC) and the photoreceptors (P). (d) High magnification shows ZnT7 staining in the pigment epithelial cells (PEL) and outer segment of photoreceptors (OS), while the inner segment of photoreceptors (IS) has none. Scale bars represent 20 μm in subpart 'a' in (a), and 5 μm in subparts 'b–d' in (a).

The outer nuclear layer showed no ZnT7 reaction (Fig. 1a; Table 1). At a higher magnification, the ZnT7 immunoreactivity was found predominately in the perinuclear regions of the cell and diffused into the peripheral cytoplasm (Fig. 1b). A fraction

Table 1
ZnT7 Immunoreactivity and AMG positive reaction in the mouse retina[a]

	ZnT7		AMG	
	Cell body	Process	Cell body	Process
Pigment epithelial cells	+++		+++	
Outer segment layer		+		−
Inner segment layer		−		+++
Outer nuclear layer	−	−	−	+
Outer plexiform layer	−	+	−	+
Amacrine cells	++	+	−	++
Bipolar cells	−	−	−	−
Horizontal cells	+	+	−	++
Inner plexiform layer		+		++
Ganglion cells	+++			+
Optic never fiber layer		++		−

Abbreviations: ZnT7, zinc transporter 7; AMG, autometallography.
[a] The intensity of immunoreactivity and AMG staining were graded as follows: (+++), strong; (++), moderate; (+), weak; (−), negative.

of the inner nuclear layer cells exhibited a moderate ZnT7 staining (Fig. 1a and c). Based on their location, these cells were presumed to be amacrine and horizontal cells.

The somata of bipolar cells in the inner nuclear layer and photoreceptors in the outer nuclear layer were void of ZnT7 immunostaining (Fig. 1c and d).

3.2. Zinc autometallographic reaction products in the mouse retina

In the AMG developer incubated sections, seven layers of retina plus the pigment epithelial layer were distinguished as seen in the ZnT7 stained sections (Fig. 2a). The distribution of AMG grains in the mouse retina found in this study was similar to the distribution of zinc in the rat retina [1]. In general, the layers stained vividly. However, the grain intensity varied significantly from layer to layer (Fig. 2a; Table 1).

The pigment epithelial cells (PEL) demonstrated the strongest AMG staining (Fig. 2e). The inner segment of the photoreceptors (IS) showed strong AMG staining visible as a brown-black band, while the outer segment was almost void of staining (Fig. 2e). From the outer nuclear layer to outer plexiform
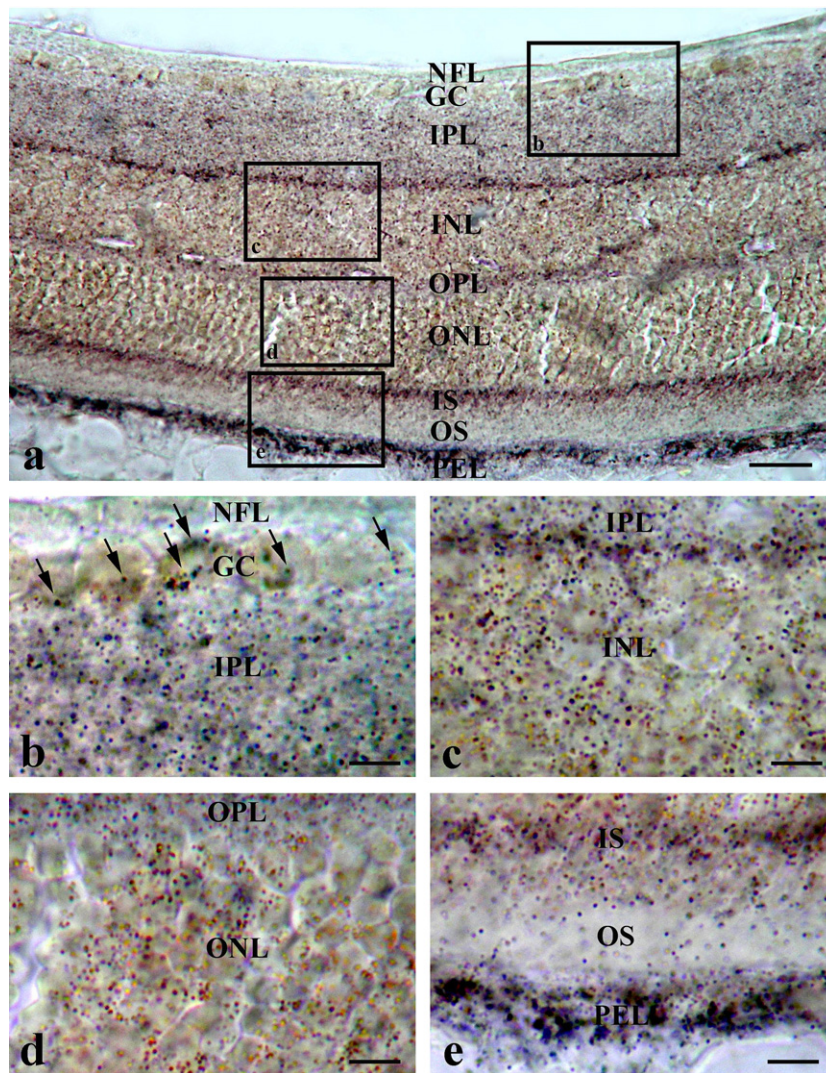
Fig. 2. Distribution of autometallographic (AMG) silver enhanced zinc–selenium nanocrystals in the mouse retina. (a) Low magnification shows a layered staining pattern of the retina; (b) AMG grains are abundant in the inner plexiform layer (IPL). Ganglion cells (GC) contained a few AMG grains (arrows). Note that the optic nerve fibers (NFL) did not stain at all; (c) a dense band of AMG grains is seen between the boundary of the inner plexiform layer (IPL) and inner nuclear layer (INL). Dispersed AMG grains are seen in the inner nuclear layer (INL); (d) AMG enhanced zinc–selenium quantum dots were seen in the outer plexiform layer (OPL) and outer nuclear layer (ONL); (e) the densest AMG staining is seen in the pigment epithelial cells (PEL) while only a few AMG grains are seen in the inner segment of photoreceptors (IS) and the outer segment of photoreceptors (OS). Scale bars represent 20 μm in subpart 'a' in (a), and 5 μm in subparts 'b–e' in (a).

layer a distinct, but diffuse, AMG staining was seen (Fig. 2d) and brown-black bands of AMG grains were distributed near the marginal regions of the inner nuclear layer (Fig. 2c). The ganglion cell layer showed a faint AMG staining (Fig. 2b). A small number of ganglion cells contained visible AMG grains in their cell bodies (Fig. 2b). The optic nerve fiber layer did not stain at all (Fig. 2b).

4. Discussion

In the present study, we have demonstrated, for the first time, the regional distribution of ZnT7 in the mouse retina. The finding that ZnT7 is localized in several retinal layers suggests that ZnT7 may be involved in zinc metabolism in the retina. We have also shown the localization of labile zinc pools in the mouse retina

by using an autometallographic in vivo tracing technique. We found similar zinc ion distribution patterns in mouse retina as has been observed in rat retina [1]. The comparison between localizations of zinc ions and zinc transporter proteins provides an important clue to further understand the retinal zinc homeostasis and physiology.

We observed that the strongest immunoreaction of ZnT7 took place in perinuclear areas of ganglion cells. This is consistent with the previous report that ZnT7 is localized in the Golgi apparatus [27]. AMG experiments reported by us and others have demonstrated that only a small amount of labile zinc ions are present in ganglion cells [1], suggesting ZnT7 may reduce labile zinc pools in the cell by mobilizing the free zinc ions into the intracellular compartments where the free zinc ions could be rapidly converted into more firmly bound zinc ions.

It is interesting that the amacrine cells exhibit a strong ZnT7 staining as a previous study has demonstrated that amacrine cell bodies show strong ZnT3 expression [36]. We found a brown-black band of silver enhanced zinc–selenium quantum dots near the marginal regions of the inner plexiform layer, the synaptic region between amacrine, bipolar and ganglion cells, which is consistent with the results reported by Akagi et al. [1]. Akagi et al. reported that ultrastructurally zinc ions were located in amacrine cell terminals with dense-core vesicles, while no zinc ions could be found in the bipolar cell terminal [1]. So in the present study, the AMG positive dense band in the inner plexiform layer is assumed to be terminals of dopaminergic amacrine cells. We also reported that ZnT3 and tyrosine hydroxylase colocalized in a fraction of sympathetic ganglion cells [45,43] and these sympathetic cells contain zinc in the Golgi apparatus [47]. Furthermore, amacrine cells are known to contain inhibitory neurotransmitters GABA and glycine. Zinc has been shown to function as a neuromodulator to inhibit synaptic transmission in interneurons in the spinal cord and cerebellum [44–46]). In retina, zinc ions may play a similar role in regulating synaptic transmission. We hypothesize that both ZnT3 and ZnT7 contribute to zinc neuromodulation in dopaminergic and/or inhibitory neuronal pathways in mouse retina.

Although the expression patterns of ZnT3 and ZnT7 and the zinc–selenium nanocrystal patterns overlaps in most regions of the mouse retina, it is important to point out that there are no overlaps in the region of the photoreceptor layer, i.e. ZnT7 is abundant in the outer segments, while chelatable zinc ions and ZnT3 are present only in the inner segments of the photoreceptor layer [36]. The colocalization of ZnT3 protein and chelatable zinc ions in the inner segments of the photoreceptor layer suggests that they may participate in mitochondrial respiration [36]. The outer segment of the photoreceptors may contain large amounts of zinc ions [30], however, these zinc ions may be tightly bound to pigments or other macromolecules and cannot be detected by AMG due to the technical limitation. Therefore, the regional distribution of ZnT7 protein in the outer segments suggests that ZnT7 may reduce the chelatale zinc concentrations by transporting zinc into Golgi network, and may have a role in packaging zinc-binding proteins in the outer segments in the retina.

We have found that both ZnT7 and chelatable zinc ions resided in the cytoplasm of pigment epithelial cells. At electron microscopic levels, zinc ions were frequently found in the Golgi apparatus in pigment epithelial cells [1]. Interestingly, both melatonin and tyrosinase, a key enzyme for the melatonin formation, are all zinc-containing proteins [37,38] and zinc deficiency could reduce the protein synthesis of cultured pigment epithelial cells [39]. Therefore, the abundant expression of ZnT7 and chelatable zinc ions supports the notion that ZnT7 is important for regulating the metabolic homeostasis in pigment epithelial cells.

Acknowledgments

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